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Evaluation of morpho-anatomical and chemical differences between varieties of the medicinal plant *Casearia sylvestris* Swartz

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ABSTRACT

Casearia sylvestris Swartz (Salicaceae) has been used in traditional medicine and its leaf extracts have been exhibited important pharmacological activities. The species presents morphological, chemical and genetic variation. Two varieties are considered due external morphological differences: *C. sylvestris* var. *sylvestris* and var. *lingua*. There are difficulties in definition of these varieties. The objective of this work is to evaluate chemical and morpho-anatomical differences between *C. sylvestris* varieties that can be applied in their distinction for pharmaceutical or botanical purposes. Transverse and paradermic sections of leaves were prepared for morpho-anatomical, histochemical and quantitative microscopy (stomatal and palisade index) analyses. Diterpene profiles of the specimens were obtained by HPLC-DAD and TLC. Morpho-anatomical analyses demonstrated significant differences between the varieties only in paradermic sections: var. *sylvestris* - polygonal epidermic cell walls and hypostomatic; var. *lingua* - rounded epidermic cell walls and amphistomatic. No differences were observed for stomatal index; palisade index was found 2.8 for var. *lingu*a and 3.9 for var. *sylvestris*. Chromatographic analyses confirmed previous results demonstrating that diterpene profile in varieties differs, with predominance of these metabolites in var. *sylvestris*. In conclusion, this work indicates that chromatographic analysis besides morpho-anatomical analysis can be applied in distinction of *C. sylvestris* varieties.

Key words: *Casearia sylvestris*, chromatography, diterpenes, morpho-anatomy.

INTRODUCTION

Casearia sylvestris Swartz (Salicaceae), known as "guaçatonga" or "erva-de-bugre", is a plant species distributed throughout South America that occurs in 22 states of Brazil, especially within the Atlantic and Amazon forests and the Cerrado biomes (Marquete 2001, Sleumer 1980). The plant has been widely used in traditional medicine for the treatment of snake bites and in wound healing, and also as an antiulcer and topical antiseptic (Hoehne 1939, Lorenzi and Matos 2002). Pharmacological studies on its leaf extracts have demonstrated

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antiulcerogenic, anti-inflammatory, antivenom, and cytotoxic (tumor cell lines) activities. Moreover, no significant toxicological effects of its ethanolic or hydroalcoholic extracts have been observed *in vitro* or following oral administration in animals (Basile et al. 1990, Borges et al. 2000, Ferreira et al. 2010, Maistro et al. 2004, Santos et al. 2010, Sertiè et al. 2000, Silva et al. 2004). The Brazilian Agency of Medicines (Agência Nacional de Vigilância Sanitária - ANVISA) recognizes the importance of the species that was included in a positive list of medicinal plants for sale in pharmacies (Brasil 2010).

Secondary metabolites isolated/identified from *C. sylvestris* include monoterpenes and sesquiterpenes (Sousa et al. 2007), *nor*-isoprenoids (Santos 2008, Wang et al. 2009a), triterpenes, lapachol, cafeic, chlorogenic and vanillic acids, flavonoids (Raslan et al. 2002), neolignans (Wang et al. 2010), ellagic and gallic acids derivatives (Silva et al. 2008a, Silva et al. 2008b). Additionally, phytochemical studies of *Casearia* species have revealed the presence of typical oxygenated tricyclic *cis*-clerodane diterpenes in which the tetrahydrofuran ring bears two acyloxy groups at C18 and C19 (Chen and Wiemer 1991, Guittet et al. 1988, Kanokmedhakul et al. 2005, Oberlies et al. 2002, Santos et al. 2010). Diterpenes of this group were isolated from *C. sylvestris*: casearins A-X and caseargrewiin F - leaves (Carvalho et al. 1998, Itokawa et al. 1990, Morita et al. 1991, Santos et al. 2010, Wang et al. 2009b); casearvestrins A-C - stems and leaves (Oberlies et al. 2002); and roots of *rel*-19*S*-acetoxy-18*R*-butanoyloxy-18,19-epoxy-6*S*-hydroxy-2*R*-(2-methylbutanoyloxy)-5*S*,8*R*,9*R*,10*S*-cleroda-3,13(16),14-triene (Espíndola et al. 2004). These compounds exhibited some important biological activities as anti-fungal, antiulcer, trypanocidal, and cytotoxicity against human tumor cell lines (Espíndola et al. 2004, Itokawa et al. 1990, Oberlies et al. 2002, Santos 2008, Santos et al. 2010).

Casearia sylvestris was first described by Swartz in *Flora Indiae Occidentalis* (1797) and Eichler and Martius (1871) included it in *Flora Brasiliensis* of Carl Friedrich Philipp von Martius, classified in Bixaceae family. Later it was classified in *Flacourtiaceae* family (Absy and Scavone 1973, Sleumer 1980). The *Angiosperm Phylogeny Group* (APG) updated the classification of Angiosperms in 2003 and some genera of *Flacourtiaceae* (e.g. *Casearia* Jacq.) were classified as Salicaceae. *C. sylvestris* Swartz (*Crateria* Bentahm section) is classified in *Samydeae* tribe, *Salicaceae* family and *Malpighiales* order (Chase et al. 2002, The Angiosperm Phylogeny Group 2003). The macromorphology of the species is described in the work of Torres and Yamamoto (1986), based on descriptions of Eichler and Martius (1871) and Sleumer (1980). The morpho-anatomical characters of the leaves were described by Absy and Scavone (1973) and Alquini and Takemori (2000).

C. sylvestris occurs in different biomes showing great morphological variability: the size, shape, texture and consistency of the leaves, the pubescence of the branches and inflorescences, the number of flowers per inflorescence, the length of peduncle, and its height - shrubs to trees (Marquete 2001, Torres and Yamamoto 1986). Sleumer (1980) considered two varieties to the species due external morphological differences, connected by intermediate forms that are difficult to distinguish: *C. sylvestris* var. *sylvestris* and *C. sylvestris* var. *lingua* (Cambess.). Silva et al. (2006) reported differences observed during collection between varieties. In Cerrado biome the specimens were shrubs, with coriaceous lighter green leaves with smaller width and length, corresponding to *C. sylvestris* var. *lingua*; unlike, specimens of Atlantic Forest were trees with different heights (always higher than 2 m), green dark leaves larger in width and length, corresponding to *C. sylvestris* var. *sylvestris*. The differences between varieties were summarized by Klein and Sleumer (1984).

Besides the macromorphological differences between the varieties, metabolism variability analyses have suggested a minor expression of diterpenes

and a greater expression of flavonoids (as rutin) in *C. sylvestris* var. *lingua*. Studies of genetic variability also indicated distinct genetic profiles for the varieties (Cavallari 2008, Silva et al. 2006).

Considering the importance of *C. sylvestris* as medicinal plant, the chemical variability observed in previous works, mainly for the bioactive diterpenes and rutin, and the difficulties to distinguish the varieties of *C. sylvestris* by macromorphological analyses in the present article, we report on the evaluation of chemical and morpho-anatomical differences between *C. sylvestris* varieties that can be applied in their distinction for botanical purposes or pharmaceutical applications.

MATERIALS AND METHODS

Materials

Leaves of *C. sylvestris* were collected at Araraquara, São Paulo State, in February 16, 2011 (in the morning). *C. sylvestris* var. *lingua* (Estação Experimental de Araraquara, Instituto Florestal, São Paulo): AGS38 (S 21.73619, W 48.17987), AGS43 (S 21.73605, W 48.17610), AGS44 (S 21.73600, W 48.17603), AGS45 (S 21.73596, W 48.17605) and AGS51 (S 21.71112, W 48.17339). *C. sylvestris* var. *sylvestris* (Universidade Estadual Paulista, UNESP Araraquara; except AGSDER): AGS101 (S 21.81466, W 48.20215), AGS102 (S 21.81466, W 48.20215), AGS103 (S 21.81631, W 4819838), AGS104 (S 21.81430, W 48.20160) and AGSDER (S 21.73596, W 4818850). Voucher specimens of AGS38, AGS43, AGS44, AGS45 AGS51, AGS101 and AGS102 were deposited with the Herbarium "Maria Eneida P. Kaufmann" of Instituto Botânico do Estado de São Paulo. AGS103 e AGS104 are clones (plant cutting) of specimens Cs400 and Cs78 that were identified as *C. sylvestris* var. *sylvestris* (Cavallari 2008).

The solvents used for extraction and TLC analyses were analytical grade (Synth®, Diadema, São Paulo, Brazil) whilst those used in HPLC analyses and solid phase extraction (SPE) were HPLC/UV grade (J. T. Baker®, Xalostoc, Mexico). Water was purified (18.1 MΩ.cm) with a Milli Q plus system (Millipore®, São Paulo, Brazil) immediately prior to use. SPE was performed using as stationary phase LiChroprep RP18 (40-63 μm; Merck®, Darmstadt, Germany). Analytical reversed-phase HPLC-DAD was performed using a Shimadzu® system (Kyoto, Japan) comprising a model Prominence® LC-20AT pump, SIL-20A autosampler, DGU-20A5 degasser, CTO-20A column oven, SPD-M20A photodiode array detector and CBM-20A communication bus module, fitted with Hypersil Gold® C18 (Thermo® Scientific, USA) column (250 × 4.6 mm, 5 μm), with control and data handling managed by LCsolution® multi-PDA software. TLC was developed in silica gel plate aluminum backed (20 x 20 cm; 200 μm) from Sorbent® Technologies. The chromatographic standards of clerodane diterpenes (caseargrewiin F, casearin B and X) were purified from leaves extracts of *C. sylvestris* and identified as published (Santos et al. 2010). Optical microscopy was developed in a Carl Zeiss® Primo Star microscope. Reagents used in histochemical analysis were prepared as described in literature (Costa 2001, Ganter and Jollés 1969-1970, Johansen 1940, Valette et al. 1998).

Morpho-Anatomical Analyses

Anatomical freehand sections (paradermic and transverse) of the middle third of fresh leaves from *C. sylvestris* were obtained with a blade. The sections were clarified with sodium hypochlorite commercial solution and wash with deionized water before stain with different reagents (Delafield hematoxylin, Astra blue, iodine green and toluidine blue) for selection of better cell wall staining and visualization of tissue structures. The anatomical descriptions were realized based on microscope visualization (40 x) of the sections on covered glass sheet and comparison with literature data (Absy and Scavone 1973, Alquini and Takemori 2000).

Histochemical Investigations

The identification and localization of secondary metabolites in the leaf transverse sections were realized using the follow reagents: Sudan III (Costa 2001, Johansen 1940) and 2.4-dinitrophenylhydrazine (Ganter and Jollés 1969-1970) for terpenes; ferric chloride (Costa 2001, Johansen 1940) and vanillin-hydrochloric acid (Costa 2001, Valette et al. 1998) for phenolic compounds.

Quantitative Microscopy

Palisade index: the average number of palisade cells beneath each epidermal cell is termed the palisade index. The paradermic sections obtained and clarified as described in Morpho-Anatomical Analyses section were mounted on covered glass sheet. Five groups of four epidermal cells were selected in each section. The palisade cells lying beneath each group were counted, being included in the count wich are more than half-covered by the epidermal cells (Evans 2002, Costa 2001).

Stomatal index: the percentage proportion of the ultimate divisions of the epidermis of a leaf which has been converted into stomata is termed the stomatal index (I):

$$I = [(S \times 100)/(E + S)] \times 100$$

Where S is the number of stomata per unit area and E is the number of ordinary epidermal cells in the same unit area. The paradermic sections (upper and lower surfaces), that were obtained and clarified as described in Morpho-Anatomical Analyses section, were stained with Astra blue and mounted on covered glass sheet (Evans 2002, Costa 2001).

Chromatographic Analyses

The following methods were developed and validated to evaluate the diterpene chromatographic profile of *C. sylvestris* leaf samples in the laboratories of Núcleo de Bioensaios, Biossíntese e Ecofisiologia de Produtos Naturais, Instituto de Química, UNESP, Araraquara-SP (data not published).

Extraction: Dried (40° C, 7 days) and powdered leaves (1.0 g) from the specimens of *C. sylvestris* were extracted twice in a test tube with 10 mL of ethyl acetate: hexane: isopropanol 91:08:01 (v/v) by sonication for 1 h. The liquid extracts were filtered, dried at room temperature and at desiccator with silica gel under reduced pressure.

High Performance Liquid Chromatography: 20.0 mg of dried extracts were dissolved in 1.0 mL of methanol: water (98:02, v/v) and submitted to further clean-up by SPE. Laboratory-prepared SPE columns (20 x 7 mm i.d.), dry packed with ca. 1.5 g of LicChroprep RP18, were activated with methanol and conditioned with methanol: water (98:02, v/v). Following application of samples, columns were eluted with 4.0 mL of methanol: water (98:02, v/v). Each eluated was dried in a desiccator with silica gel under reduced pressure, dissolved in methanol (1.0 mL), and filtered through PVDF membranes (0.45 μm) prior to HPLC analysis. The standards of clerodane diterpenes (1.0 mg) were dissolved in methanol HPLC grade (1.0 mL) and filtered through PVDF membranes (0.45 μm). Aliquots of 20 μL were injected onto Hypersil Gold® C18 column (250 × 4.6 mm, 5 μm), which were eluted with a mixture of methanol, acetonitrile and water, initially at 22:44:34 (v/v), changing by linear gradient to 47:53:00 over 42 min, isocratically with 47:53:00 for 5 min, and finally isocratically with 22:44:34 for 10 min (equilibration). The solvent flow rate was 0.8 mL/min. Detection was at 200-700 nm for all samples. Caseargrewiin F, casearin B and X (1.0 mg/mL; methanol) were used as standards.

Casearin-like diterpenes present two different patterns of conjugated double bond in their lateral chain (C11-C16) - C12(Z or *E*)/C14 or C13(16)/C14 - and their UV spectra present one band and λ_{max}= 231-238 or 221-228 nm, respectively (Carvalho et al. 2009, Espíndola et al. 2004, Itokawa et al. 1990). Thus, we suggested that peaks with UV spectra with one band and λ_{max}= 221-228 or 231-238 nm were relative to the casearin-like diterpenes.

Carvalho et al. (2009) analyzed different organs of *C. sylvestris* with similar approach and results from HPLC-DAD analyses were confirmed by ^{1}H NMR and TLC data. Moreover, five casearin-like diterpenes isolated from leaves of *C. sylvestris* were identified in its extract and presented peaks with t_R in the range of 10-30 min in the same HPLC conditions (Santos 2008). Additionally, as observed by Carvalho et al. (2009), peaks with a second band in the UV spectra with λ_{max} of c.a. 280 nm, indicate the presence of clerodane diterpenes with a aromatic ester substituent, as reported in the literature (Beutler et al. 2000). Thus, clerodane diterpenes were identified in the extract chromatograms on the basis of UV spectra of the peaks. Additionally, the comparison of retention time (t_R) and UV spectra of peaks from standard and extract chromatograms was realized.

Thin Layer Chromatography: 8.0 mg of dried extracts and 1.0 mg of caseargrewiin F and casearin B were dissolved in 1.0 mL of ethyl acetate. TLC was developed in silica gel plate aluminum backed (20 x 20 cm; 200 μm) using hexane: ethyl acetate: isopropanol 70:28:02 (v/v) as eluent and sulfuric anisaldehyde as spray reagent.

RESULTS

The macromorphological differences between the varieties of *C. sylvestris* reported were evident during the collection and the specimens were classified as *C. sylvestris* var. *lingua* or *C. sylvestris* var. *sylvestris* as described by Sleumer (1980) and Klein and Sleumer (1984).

In the morpho-anatomical study, the best visualization in transverse sections was achieved with dual staining: Delafield hematoxylin followed by green iodine. No significant anatomical differences were observed in transverse sections for the varieties. Morpho-anatomical characters of the leaves were in agreement with literature (Absy and Scavone 1973, Alquini and Takemori 2000). In the midvein region of leaf sections we observed typical cells of xylem, phloem, collenchyma and parenchyma, in addition to fibers associated with vascular bundles (Figure 1a). Both upper and lower epidermises were uniseriate, presenting a lower number of simple nongladular trichomes. The mesophyll is asymmetrical and heterogeneous with two (rarely three) layers of palisade parenchyma and several layers of spongy parenchyma with irregular cells in shape and size. The air spaces (meatus) occur in small proportion in spongy parenchyma (Figure 1b). We also noted the presence of spiral vessels, oil glands, druses and prismatic crystals (calcium oxalate) (Figure 2).

For the paradermic sections, the best stain reagent was Astra blue. In this case we observed significant differences between the varieties. The epidermic cell walls are polygonal in *C. sylvestris*

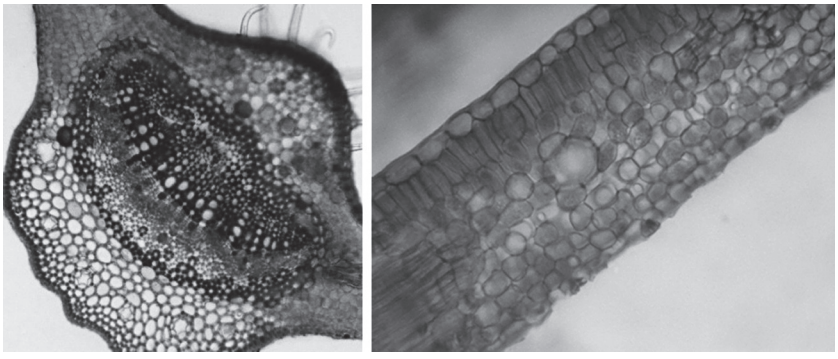

Figure 1 - Tranverse section of the midvein region from the leaf of *C. sylvestris* var. *lingua* **(a)** and tranverse section of the region between the midvein and the margin from the leaf of *C. sylvestris* var. *sylvestris* **(b)**.

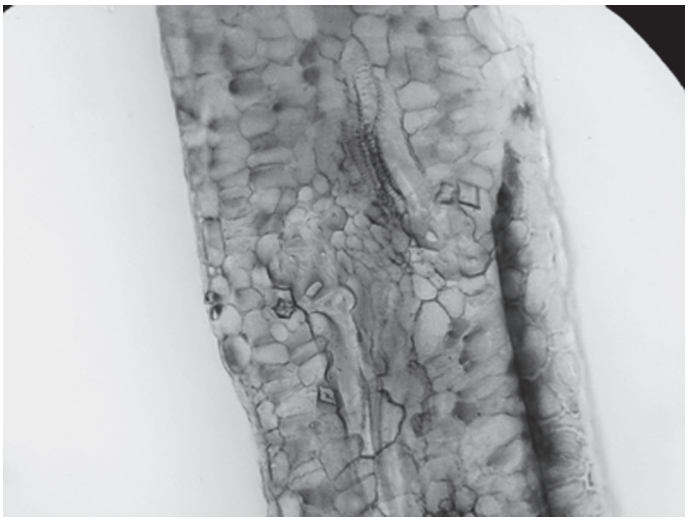

Figure 2 - Transverse section of the region between the midvein and the margin from the leaf of *C. sylvestris* var. *lingua*.

var. *sylvestris* and rounded in *C. sylvestris* var. *lingua* (Figure 3). Moreover, *C. sylvestris* var. *sylvestris* is hypostomatic and *C. sylvestris* var. *lingua* is amphistomatic. The stomata are paracytic in both varieties.

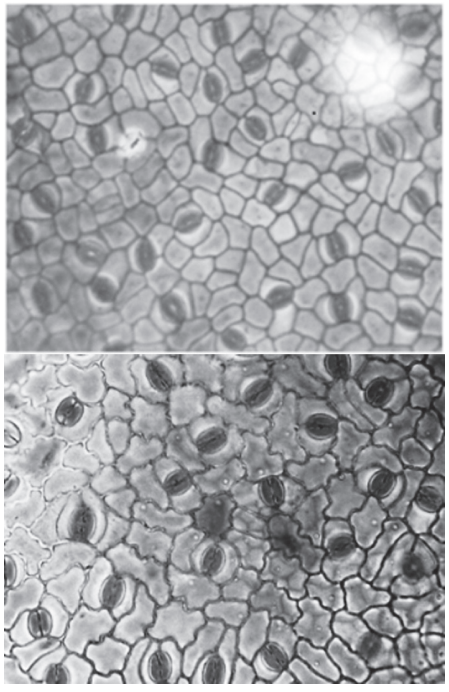

Figure 3 - Paradermic sections (lower epidermis) from the leaf of *C. sylvestris* var. *sylvestris* **(a)** and var. *lingua* **(b)**.

Histochemical reagents did not differentiate the varieties and the results were positive for both terpenes and phenolics (all reagents tested).

The results of quantitative microscopy are in Tables I and II. The values of stomatal index for specimens of *C. sylvestris* var. *sylvestris* presented great variation: 11.9 to 20.7 % (standard deviation = 3.4). In the case of *C. sylvestris* var. *lingua* specimens these values were more homogeneous (standard deviation = 0.2). The mean value of stomatal index for both varieties was similar: 15.9 (var. *sylvestris*) and 16.4 % (var. *lingua*). On the other hand, palisade index was found 2.8 for var. *lingua* and 3.9 for var. *sylvestris*, demonstrating that this index should be useful in differentiation of *C. sylvestris* varieties.

TLC analyses provided information about clerodane diterpenes profile of the extracts. Caseargrewiin F (R_f = 0.23) was identified by TLC (Figure 4) in the extracts of specimens AGS101, AGS102, AGS103, AGS104 (var. *sylvestris*), AGS44, AGS45 and AGS51 (var. *lingua*), while casearin B (R_f = 0.26) was not identified in any extract. Moreover, TLC profiles of var. *sylvestris* specimens are similar, except for AGSDER. Unlike, specimens of var. *lingua* presented different TLC profiles, except for AGS44

TABLE I
Stomatal index for specimens of *C. sylvestris*.

SPECIMEN	STOMATAL INDEX (%)	
	LOWER EPIDERMIS	UPPER EPIDERMIS
var. *sylvestris*		
AGS103	15.6	-----
AGS104	13.8	-----
AGSDER	11.9	-----
AGS101	20.7	-----
AGS102	17.7	-----
mean (sd[1])	*15.9 (3.4)*	-----
var. *lingua*		
AGS 38	16.3	10.3
AGS 43	16.5	9.5
AGS 44	16.1	9.7
AGS 45	16.7	10.1
AGS 51	16.4	9.2
mean (sd)	*16.4 (0.2)*	*9.8 (0.4)*

[1]standard deviation.

TABLE II
Palisade index for specimens of *C. sylvestris*.

SPECIMEN	PALISADE INDEX
var. *sylvestris*	
AGS103	3.7
AGS104	4.3
AGSDER	3.8
AGS101	3.9
AGS102	3.8
mean (sd[1])	*3.9 (0.2)*
var. *lingua*	
AGS 38	3.1
AGS 43	3.2
AGS 44	2.5
AGS 45	2.6
AGS 51	2.6
mean (sd)	*2.8 (0.3)*

[1]standard deviation.

and AGS45. These specimens have TLC profile similar to specimens of var. *sylvestris*.

Table III presents results of HPLC-DAD analyses for the ten specimens considering number of peaks and area of selected peaks based on

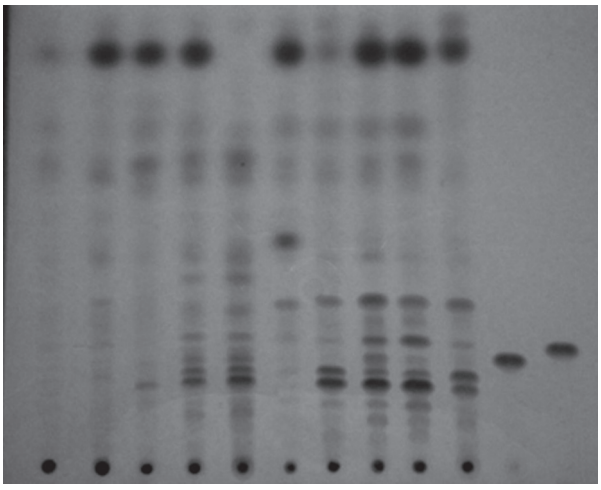

Figure 4 - Thin layer chromatographic profile (silica; hexane: ethyl acetate: isopropanol 70:28:02; sulfuric anisaldehyde) of extracts from leaves of *C. sylvestris*. Samples (from left to right): AGS38, AGS51, AGS43, AGS45, AGS44 (var. *lingua*); AGSDER, AGS102, AGS101, AGS103, AGS104 (var. *sylvestris*); caseargrewiin F, casearin B (clerodane diterpenes).

UV spectra/λ_{max}. Figure 5 shows representative chromatograms of both varieties and the structures of diterpene standards. As observed in the example of Figure 5, there is a greater total area of peaks with $t_R < 5$ min in the chromatograms of specimens of *C. sylvestris* var. *lingua* and AGSDER (var. *sylvestris*) than in chromatograms of AGS101-104 (var. *sylvestris*). On the other hand, the total area of peaks with λ_{max}= 221-228 or 231-238 nm is greater in chromatograms of AGS101-104 (Table III).

We classified the specimens in three groups on the basis of the observed common main peaks (peaks with larger area), identification of clerodane diterpenes (caseargrewiin F, casearin B and X) and predominance of peaks with UV spectrum with λ_{max}= 221-228 or 231-238 nm. The diterpene standards were identified in the extracts by comparison of their t_R and UV spectra. Tables IV-VI present data from chromatograms of the three groups: group 1 - AGS38, AGS44-45 (var. *lingua*); group 2 - AGS43 and AGS51 (var. *lingua*); group 3 - AGS101-104 (var. *sylvestris*). AGSDER demonstrated a chromatographic profile with characteristics of different groups.

TABLE III
HPLC-DAD analyses data of leaf extracts from different specimens of *C. sylvestris*. The number of peaks and total area of selected peaks based on UV spectra/λ_{max} are presented.

specimen	λ_{max}= 221-228 nm		λ_{max}= 231-238 nm		λ_{max}= 221-228 nm and c.a. 280 nm		λ_{max}= 231-238 nm and c.a. 280 nm	
	total area	n. of peaks	total area	n. of peaks	total area	n. of peaks	total area	n. of peaks
var. *sylvestris*								
AGS101	1,487,789	4	205,577,366	29	10,618,521	6	3,406,177	2
AGS102	1,094,944	3	142,165,961	25	2,437,009	3	8,897,657	4
AGS103	3,204,639	3	241,678,210	33	355,487	1	977,877	1
AGS104	2,070,050	4	223,721,237	24	5,375,576	5	12,867,946	2
AGSDER	7,808,800	13	7,670,449	3	2,537,429	5	--	--
var. *lingua*								
AGS38	25,490,047	22	4,976,442	3	989,125	2	--	--
AGS43	2,410,937	6	52,521,251	17	1,821,007	4	--	--
AGS44	55,220,208	23	8,368,142	2	2,633,624	6	342,820	1
AGS45	34,518,773	23	31,243,011	2	4,585,270	5	--	--
AGS51	790,020	8	21,440,354	16	--	--	--	--

TABLE IV
Data from HPLC-DAD analyses of specimens AGS38, AGS44 and AGS45. Retention time, area and λ_{max} of selected peaks (t_R > 10 min; area > 10^6 in at least one specimen of the group) are presented.

AGS38			AGS44			AGS45		
t_R (min)	area	λ_{max}[1] (nm)	t_R (min)	area	λ_{max} (nm)	t_R (min)	area	λ_{max} (nm)
10.1	*214,038*[3]	220	10.1	3,544,160	223	10.0	4,007,308	223
12.0	*230,733*	220	12.1	2,779,635	223	12.0	3,827,383	223
13.9	1,441,518	223	13.9	1,772,768	223	13.8	2,932,737	223
15.0	6,096,411	239	15.0	15,390,898	238	14.9	16,979,443	238
15.6	2,749,524	223	15.6	6,391,880	223	15.5	7,712,235	222
16.8[2]	4,072,823	237	**16.9**[2]	17,521,082	237	**16.7**[2]	14,263,568	237
18.2	2,416,173	223	18.2	1,976,262	223	18.0	3,328,239	223
19.7	3,297,201	223	19.7	655,312	223	19.6	1,541,503	223
---	---	---	---	---	---	19.9	1,945,180	223
22.7	*881,097*	223	22.7	*682,384*	223	22.5	1,182,339	223
27.6	2,022,085	223	27.6	6,344,883	221	27.4	7,792,553	220
31.6	1,672,354	223	31.7	*208,153*	223	31.5	*340,354*	223
32.5	2,418,817	223	32.7	2,024,131	223	32.5	2,625,140	223
---	---	---	---	---	---	35.0	1,641,322	---
37.1	1,007,356	223	37.1	*44,921*	220	36.9	*245,582*	---
37.9	1,036,496	223	37.8	*14,956*	220	37.6	*55,241*	---

[1]all spectra presented one band.
[2]peak identified as caseargrewiin F (t_R= 16.9 min; λ_{max}= 235 nm).
[3]values in italic indicates that peak area< 10^6 for this specimen.

TABLE V

Data from HPLC-DAD analyses of specimens AGS43 and AGS51. Retention time, area and λ_{max} of selected peaks (t_R > 10 min; area >10^6 in at least one specimen of the group) are presented.

AGS43			AGS51		
t_R (min)	area	λ_{max}[1] (nm)	t_R (min)	area	λ_{max} (nm)
10.7	2,444,373	234	10.7	*551,446*	231
12.8	2,667,364	234	---	---	---
13.4	2,884,543	234	---	---	---
14.5	1,364,805	233	14.3	*197,898*	231
15.0	6,532,573	238	---	---	---
15.9	14,370,840	234	16.2	1,322,891	234
16.4	2,578,648	234	---	---	---
16.8[2]	2,388,160	236	**16.6**[2]	2,111,535	237
18.0	1,430,820	231	17.7	302,861	233
19.1[2]	2,020,369	234	**18.8**[2]	2,741,439	234
---	---	---	20.1	6,083,237	234
20.7	6,040,123	234	---	---	---
21.6[2]	1,475,264	233	**21.3**[2]	*317,727*	232
23.3	1,245,694	231	23.0	1,759,548	234
28.2	2,392,835	234	---	---	---
29.8	1,090,286	227	29.6	*103,058*	227
---	---	---	31.9	1,228,921	234
33.1	5,187,343	230	32.8	1,505,320	234
35.1	1,660,173	259	34.9	*184,321*	258
35.9	1,254,948	233	35.7	*335,305*	230

[1]all spectra presented one band.

[2]peaks identified as caseargrewiin F (t_R= 16.9 min; λ_{max}= 235 nm), casearin B (t_R= 19.0 min; λ_{max}= 233 nm) or casearin X (t_R= 21.4 min; λ_{max}= 234 nm).

[3]values in italic indicates that peak area< 10^6 for this specimen.

TABLE VI

Data from HPLC analyses of specimens AGS101-104. Retention time, area and λ_{max} of selected peaks (t_R > 10 min; area >10^7 in at least one specimen of the group) are presented.

AGS101			AGS102			AGS103			AGS104		
t_R (min)	area	λ_{max}[1] (nm)	t_R (min)	area	λ_{max} (nm)	t_R (min)	area	λ_{max} (nm)	t_R (min)	area	λ_{max} (nm)
11.5	51,753,295	232	11.5	41,903,478	234	11.4	63,905,455	232	11.5	35,911,329	232
14.9	*2,274,634*	237	15.1	*4,923,216*	235	14.9	12,408,073	238	15.0	10,271,162	235
16.3	*8,262,055*	234	16.3	27,280,273	231	16.2	19,190,603	233	16.1	51,263,748	232
16.7[2]	*3,172,351*	235	**16.7**[2]	*2,946,438*	237	**16.6**[2]	11,387,481	237	**16.6**[2]	*6,483,562*	237
18.9[2]	*16,510,122*	233	**18.9**[2]	*4,863,225*	234	**18.8**[2]	31,961,624	232	**18.8**[2]	14,334,030	233
20.2	39,245,812	232	20.2	20,544,230	233	20.2	30,553,286	232	20.1	40,426,106	232
21.5[2]	*602,260*	234	**21.6**[2]	*2,524,503*	234	**21.4**[2]	*3,562,338*	235	**21.5**[2]	*2,283,234*	234
23.1	15,510,748	234	23.1	*5,300,509*	235	23.1	11,112,349	234	23.0	11,399,559	234
32.0	14,119,935	234	31.9	*1,418,784*	235	31.9	*7,955,250*	234	31.9	*1,592,905*	234
33.0	10,990,895	234	32.8	*5,918,022*	234	32.8	18,209,028	232	32.8	*2,665,132*	234

[1]all spectra presented one band.

[2]peaks identified as caseargrewiin F (t_R= 16.9 min; λ_{max}= 235 nm), casearin B (t_R= 19.0 min; λ_{max}= 233 nm) or casearin X (t_R= 21.4 min; λ_{max}= 234 nm).

[3]peak area values in italic highlights that peak area< 10^7 for this specimen.

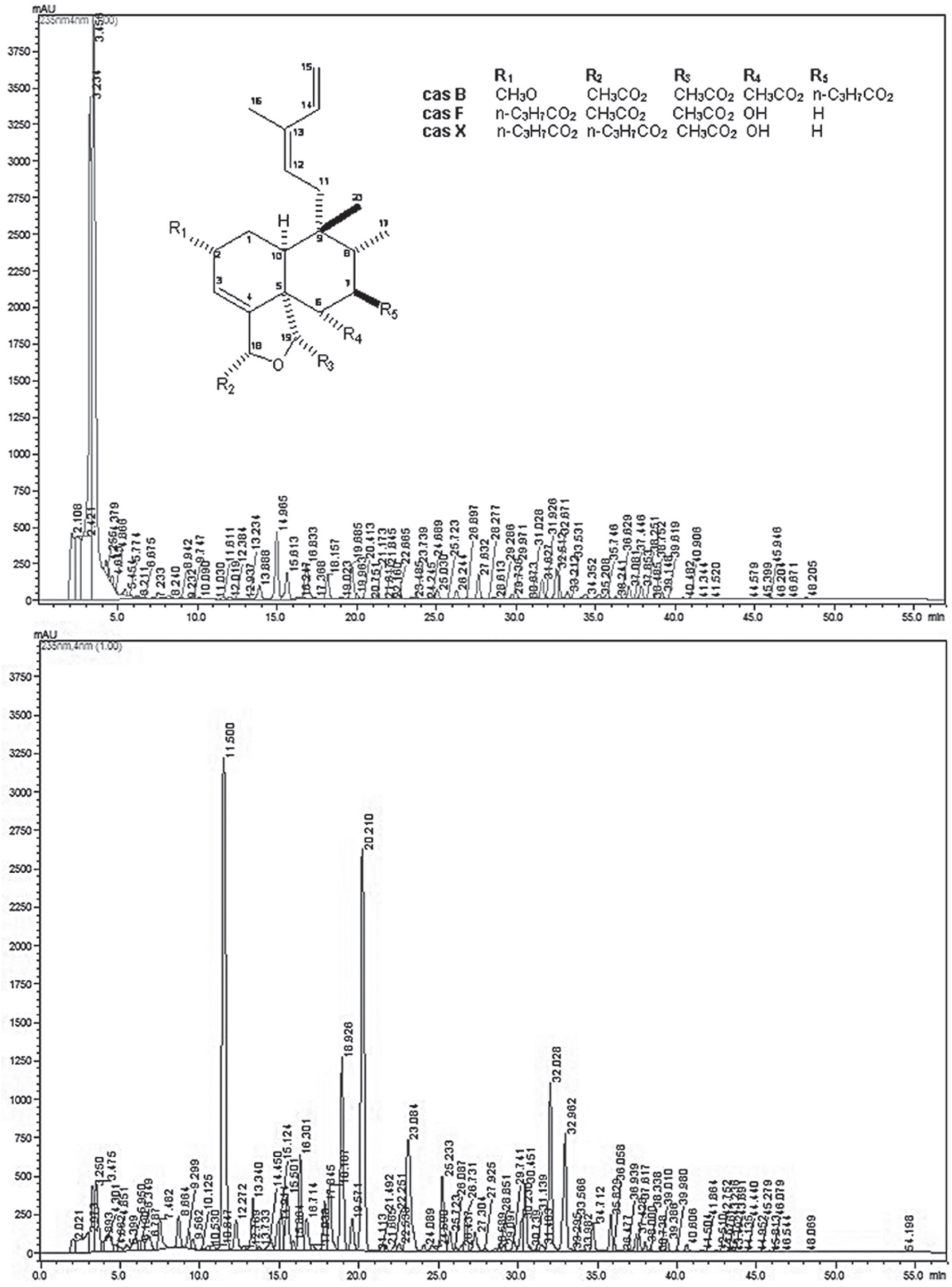

Figure 5 - Representative high performance liquid chromatography (C18; methanol: acetonitrile: water gradient; 235 nm) profile of extracts from leaves of *C. sylvestris* - specimens AGS38 var. *lingua* (top) and AGS101 var. *sylvestris* (bottom). Structures of diterpenes standards are presented: caseargrewiin F (cas F), casearins B and X (cas B and cas X).

DISCUSSION

The morpho-anatomical comparison between the varieties revealed differences only in the paradermic sections. The shape of epidermic cell walls (polygonal in var. *sylvestris* and rounded in var. *lingua*) and stomata distribution (var. *sylvestris* is hypostomatic and var. *lingua* is amphistomatic) are characteristics that can be applied in the differentiation of the varieties as well as the palisade index. According Costa (2001) the palisade index is less affected by environmental factors than stomatal index. These results are interesting for the pharmaceutical quality control application because the analysis of the powdered drug will lead to the identification of the varieties. Obviously, the assays will be applied to more representative number of specimens from different environments or regions to be validated.

Data from HPLC-DAD analyses (t_R and UV spectrum) allowed to classify the specimens in three groups. In the first group - AGS38, AGS44 and AGS45 (var. *lingua*) - we found the following common characteristics: identification only of caseargrewiin F, predominance of peaks with λ_{max}= 221-228 nm (22-23 peaks and total peak area of 22-55 x 10^6), and seven common main peaks with area > 10^6 (Tables III and IV). In TLC analyses caseargrewiin F was not identified in AGS38 probably due its minor concentration as demonstrated by HPLC analysis (small peak area).

In the second group - AGS43 and AGS51 (var. *lingua*) - caseargrewiin F, casearins B and X were identified. In the TLC analyses, diterpenes were not identified as discussed above. The peaks with λ_{max}= 231-238 nm have greater area (16-17 peaks and total peak area of 21-53 x 10^6) than peaks with λ_{max}= 221-228 nm (6-8 peaks and total peak area of 0.8-2.4 x 10^6) (Table III). Finally, there are five common main peaks with area > 106 (Table V) in their chromatograms.

The third group includes AGS101-104 (var. *sylvestris*). In this group the peaks with λ_{max}= 231-238 nm predominate notably in number of peaks (24-33) and total peak area (142-242 x 106) in comparison with all specimens of var. *lingua* (Table III). The three diterpenes were identified in these specimens and they have six common main peaks with area > 4 x 10^6 (Table VI). In the TLC analysis, casearin B was not identified as discussed above.

The specimen AGSDER presented characteristics of different groups. Its chromatogram demonstrated that peaks with λ_{max}= 221-228 nm predominate in number (13) but no differences were observed for peak total area (8 x 10^6) considering the two ranges of λ_{max}. Casearin B and caseargrewiin F were identified in HPLC-DAD analysis.

The results obtained suggest the predominance of clerodane diterpenes, especially with λ_{max}= 231-238 nm, in *C. sylvestris* var. *sylvestris*. Moreover, there is a greater total area of peaks with t_R< 5 min in the chromatograms of specimens of *C. sylvestris* var. *lingua* and AGSDER (var. *sylvestris*) than in chromatograms of AGS101-104 (var. *sylvestris*) indicating greater concentration of polar compounds (e.g. phenolics) as demonstrated in previous work (Cavallari 2008, Silva et al. 2006). AGSDER was in an urban area subjected to pollutants and it may be correlated with its different chemical pattern.

As morpho-anatomical analyses, the chemical analyses by HPLC-DAD have to be applied to greater number of specimens from different environments or regions to give more conclusive results.

In conclusion, this work indicates that chemical analyses, mainly HPLC-DAD, besides morpho-anatomical analyses of paradermic sections and palisade index can be applied in distinction of *C. sylvestris* varieties for botanical or pharmaceutical purposes.

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RESUMO

Casearia sylvestris Swartz (Salicaceae) é uma planta utilizada na medicina tradicional, cujos extratos de folhas demonstraram importantes ações farmacológicas. A espécie apresenta variação morfológica, genética e química. Duas variedades são consideradas devido a diferenças morfológicas: *C. sylvestris* var. *sylvestris* e var. *lingua*. Há dificuldades na definição destas variedades. O objetivo deste trabalho é avaliar diferenças morfo-anatômicas e químicas entre as variedades de *C. sylvestris* que permitam sua diferenciação com aplicação farmacêutica ou botânica. Seções transversais e paradérmicas de folhas foram preparadas para análises morfo-anatômicas, histoquímicas e microscopia quantitativa (indices de estômatos e paliçada). Análises cromatográficas (CLAE-DAD e CCD) foram realizadas para obter o perfil de diterpenos clerodânicos. Os resultados das análises morfo-anatômicas demonstraram diferenças significativas entre as variedades somente em cortes paradérmicos: var. *sylvestris* - paredes celulares epidérmicas poligonais e hipoestomática, var. *lingua* - paredes celulares epidérmicas arredondadas e anfiestomática. Os índices de estômatos não revelaram diferenças; os valores dos índices de paliçada foram de 2,8 para var. *lingua* e 3,9 para var. *sylvestris*. As análises cromatográficas confirmaram resultados prévios, demonstrando predomínio de diterpenos na var. *sylvestris*. Este trabalho sugere que análises cromatográficas e morfo-anatômicas podem ser ferramentas aplicáveis na distinção das variedades da espécie.

Palavras-chave: *Casearia sylvestris,* cromatografia, diterpenos, morfo-anatomia.

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